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Population genetic consequences of the reproductive system in the liverwort *Mannia fragrans*

Zsófia Hock · Péter Szövényi · Jakob J. Schneller · Edwin Urmi · Zoltán Tóth

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Abstract Ecological factors affecting reproduction and dispersal are particularly important in determining genetic structure of plant populations. Polyoicous reproductive system is not rare in bryophytes; however, to date, nothing is known about its functioning and possible population genetic effects. Using the liverwort *Mannia fragrans* as a model species, the main aims of this study were to separate the relative importance of the components of the polyoicous reproductive system and to assess its consequences on the genetic structure of populations. High sex expression rates increasing with patch size and strongly female-biased sex ratios were detected. Additional input into clonal growth after production of sex organs was found in males compared to females. Similar clonal traits of the rare bisexual and asexual plants and preference toward newly colonized patches suggest that selection prefers colonizers that first develop organs of both sexes, hence ensuring sexual reproduction when no partner is present. Despite frequent spore production, ISSR markers revealed low genetic diversity, probably resulting from the effective clonal propagation of the species and frequent crossing between genetically identical plants. The presence of numerous rare alleles and unique recombinant haplotypes indicates occasional recombination and mutation. Effective spreading of new haplotypes is probably hampered however by large spore size. Since populations are small and isolated, such haplotypes are probably continuously eliminated by genetic drift. These results suggest that although both sexual and asexual reproductions seem to be effective, asexual components of the reproductive system play a greater role in shaping the genetic composition of the populations.

Keywords Cost of reproduction · Genetic diversity · Polyoicous · Reproductive ecology · Sex expression · Sex ratio

Z. Hock (✉) · P. Szövényi · J. J. Schneller · E. Urmi
Institute of Systematic Botany, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland
e-mail: hockzsofia@hotmail.com; szovenyi@systbot.unizh.ch

Z. Hock · Z. Tóth
Department of Plant Taxonomy and Ecology, Eötvös Loránd University, Budapest, Hungary

P. Szövényi
Department of Biology, Duke University, Durham, NC, USA

Introduction

Polyoicous reproductive system (i.e., populations consisting of bisexual gametophytes and unisexual gametophytes of both sexes) occurs in 7.5% of all moss species (Wyatt and Anderson 1984) and in about 5% of the liverwort species of the British Isles (based on Paton 1999). However, to date, only descriptive data mostly related to taxonomy exist

about polyoecy in bryophytes (e.g., Wyatt and Anderson 1984), whereas the functioning, dynamics, and significance in population biology of this reproductive system are poorly understood. Nothing is known, for instance, about the processes that maintain bisexual individuals in the populations (Damsholt 2002). Since sex expression rates, distribution and proportion of sexual (including male, female, and bisexual plants) and asexual plants, within populations may greatly influence reproductive success, it is especially intriguing to define the role and importance of each of these components in the life of populations. In addition, life-history characteristics, such as reproductive system, and frequency of sexual reproduction may considerably alter the extent and partitioning of genetic variability (Loveless and Hamrick 1984) as well.

Sex expression rates and the relative frequencies of male and female plants are important, since lack of one sex or skewed sex ratios reduce or hamper successful fertilization. Sex expression depends on several parameters including internal factors and environmental features such as patch size (McLetchie and Puterbaugh 2000). Larger patches have greater microsite diversity and the presence of more potential partners may stimulate sex expression as well (Chopra and Sood 1973).

In bryophytes, sex ratios are very often skewed, with prevalence of females among unisexual species (Bisang and Hedenäs 2005). Underlying causes range from differential germination and survival to differing environmental requirements, tolerance, and clonal growth patterns of sexes (Bisang and Hedenäs 2005). Life-history theory predicts that in case of limited resources, a negative correlation should exist between resources invested in current reproduction and future survival, growth, and reproduction (Stearns 1989). Although cost of reproduction is supposed to be relatively easy to measure in bryophytes due to their low ability to compensate it (lack of below-ground structures specialized for storage), attempts to assess it are sparse (Bisang and Ehrlén 2002; Rydgren and Økland 2003; Pohjamo and Laaka-Lindberg 2003). Differential costs of sexuality in males and females have been found (Stark 2002a) and higher costs were detected in fertilized than in unfertilized females (Rydgren and Økland 2003).

Numerous bryophytes, especially short-lived species specialized on temporarily available microhabitats, produce large amounts of both sexual and asexual propagules (During 1992). Yet, in most species, little is known about the relative success of the two ways of reproduction. If polyoecy and frequent sporophyte production are coupled with extensive asexual propagation, it is difficult to predict the extent to which the two different reproductive modes contribute to the genetic composition of populations. If asexual propagation is more important, within-population genetic variability is expected to be low, populations being composed of only a few clones. It is, however, important to consider that fertilization between genetically identical thalli and intra-gametophytic selfing of bisexual individuals results in spores equivalent to clonal propagules (Wyatt et al. 1989).

The first objective of the present study was to quantitatively compare the role of asexual and sexual elements of the reproductive biology in a polyoicous model species, in order to better understand the functioning of this unexplored system. More specifically, sex expression rates, and sex-ratio patterns were estimated. Cost of reproduction and regeneration from vegetative fragments were assessed to detect potential differences in the reproductive investment of the different sexual states. The second aim was to test to what extent the different elements of the reproductive system (sexual states, sexual vs. asexual elements) influence genetic structure within populations. ISSR markers were applied to distinguish between the relative success and importance of sexual versus asexual reproduction.

Materials and methods

Model species

Mannia fragrans (Balb.) Frye and Clark is a thallose, xerophilous liverwort growing on bare soil in open, exposed patches of dry grasslands. The species is polyoicous, although the proportion of bisexual thalli in populations is often very low (pers. obs.), and several populations seem to lack such plants (Damsholt 2002). In addition to regular and abundant sporophyte production every spring, clonal propagation by fragmentation is also very intensive in the species (Damsholt 2002, pers. obs.). In the study area, populations of *Mannia fragrans* are geographically isolated resulting from fragmented occurrence of

grassland communities. Although spore production is frequent and regular, large size of spores (60–80 μm, Damsholt 2002) probably hampers effective long-range dispersal. A former isozyme study on *M. fragrans* populations reported genetically polymorphic populations from the investigated region (Odrzykoski and Szweykowski 1981).

Study areas

Three Hungarian populations of *Mannia fragrans* were sampled (Fig. 1). A population was defined as a group of patches of the species occurring at an isolated station. Population 1: Vértes Mountains (N 47°31′21″, E 18°29′57″), Population 2: Mecsek Mountains (N 46°06′09″, E 18°12′27″), Population 3: Szent György Hill (N 46°49′39″, E 17°29′55″), Hungary. These populations are surrounded by forests and agricultural landscape, with the closest populations of the species being 10–30 km away. Populations 1, 2, and 3 are hereafter referred to as P1, P2, and P3, respectively.

Sampling occurred three times, in the main vegetation periods of the grasslands: (1) November 2004, before spore production, (2) April 2005, immediately following spore dispersal, and (3) November 2005. P3 was sampled only in November 2005.

Sampling and DNA analysis

At each locality, all patches of individuals were sampled, marked, photographed, and plotted on a map. The number of individuals growing in each patch (Table 1), as well as sex ratios per patch was noted. For the genetic analysis, a sample of 1–10 plants/patch (depending on the size of the patch) was taken from each patch. (Table 2).

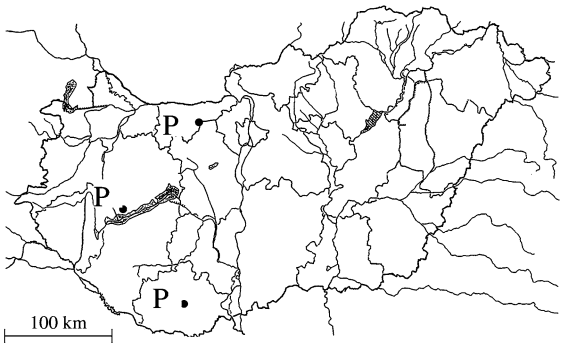

Fig. 1 Location of the three populations investigated

The collected individuals were manually cleaned under a dissecting microscope. In order to exclude potential fungal contaminants, which are reported as being common in liverwort thalli (Read et al. 2000), rhizoids and ventral scales were thoroughly removed and only the green, apical parts were used in the genetic analyses. To remove small soil particles, each plant was put in deionized water and stirred for 5–10 min using a magnetic stirrer.

DNA was extracted using the QIAGEN DNeasy Plant Mini Kit, following the manufacturer's instructions with a modified final step because of the small amounts of plant material. In order to concentrate the samples, instead of washing and incubating the samples with AE buffer, we washed them twice with 100 μl ddH_2O. Water was evaporated using a DNA 120 SpeedVac vacuum concentrator and the DNA diluted with 30 μl AE buffer. ISSR markers were chosen because of their reliability and success in other population studies (Wolfe and Liston 1998; Gunnarsson et al. 2005; Hassel et al. 2005). During preliminary studies only three primers yielded satisfactory results (Table 3).

Three microliters of DNA (2 ng/μl) was added to a reaction mixture containing 9.95 μl ddH_2O, 3.35 μl 25 mM $MgCl_2$, 2.5 μl 10× buffer (100 mM

Table 1 Number and size range of patches/population

Number of patches	
Population 1	
Small	9
Large	1
Population 2	
Small	10
Large	5

Small patch = 1–100 individuals; large patch = more than 100 individuals. Patch sizes were almost constant during the term of the study

Table 2 Number of samples used for the genetic study

Sampling	Population 1	Population 2	Population 3
1	42	40	0
2	39	30	0
3	45	28	27

Table 3 Primers used in the study

Primer name	Sequence (5′-3′)	Annealing temp. (°C)	No. of loci	No. of polymorphic loci
UBC 834	AGAGAGAGAGAGAGAGYT	45	25	25
UBC 888	BDBCACACACACACACA	46	20	20
UBC 889	DBDACACACACACACAC	51	22	21

Y = C, T; B = C, G, T; D = A, G, T

Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin), 4 µl 1.25 mM dNTPs, 2 µl primer, and 0.2 µl 5u/µl *Taq* polymerase (Sigma). DNA was amplified on a Biometra T1 thermocycler using the following program: 4 min at 94°C and 35 cycles of 1 min at 94°C, 2 min at primer specific annealing temperatures (cf. Table 3), and 2 min at 72°C followed by a final 7-min extension at 72°C. Amplification products were visualized by agarose gel electrophoresis (1.4%). Bands were scored manually and a table of presence/absence of ISSR bands was established. PCR reaction and/or extraction was repeated in case of problematic samples or those yielding very different patterns.

Sex expression, sex ratios, clonal traits, and testing for outcrossing

To estimate the effect of formation of sex organs on morphological traits/clonal growth of the thallus, from all sampling dates altogether 385 thalli from P1 and P2 were cultivated for 2 months in moistened, closed plastic bags under ca. 1,000 µmol PAR m^{-2} s^{-1}. Since most plants from P3 were killed by a fungal infection, no estimations were done for this population. Patch size was noted for each of the analyzed plants to test its effect on sex expression rates and clonal traits. Patch sizes were almost constant during the term of the study. Prior to cultivation, the sex state of each plant was determined. After 2 months, an additional three parameters were recorded for each cultivated individual: number of bifurcations (B) and number of sub-apical (A) and lateral branches (L, Fig. 2). To test the cost of realized sexual reproduction in females, 14 female plants without sporophytes and 28 plants with mature, dehisced sporophytes from the second sampling date were cultivated in the same way. The above-mentioned parameters were then noted for these plants as well.

To gain information about sex ratios and overall rates of sex expression, sex state of further 1,350 thalli from all populations and sampling dates was established (altogether 1,735 plants were analyzed).

In order to estimate outcrossing, non-dehisced sporophytes of 17 carpophores from P2 were analyzed using ISSR markers along with the 14 mother plants belonging to these sporophytes.

Asexual reproduction by fragmentation was investigated by cultivating the following cleaned 1–1.5 cm fragment types for 1 year on sterilized soil: 232 green fragments including apex, 206 brownish but not yet

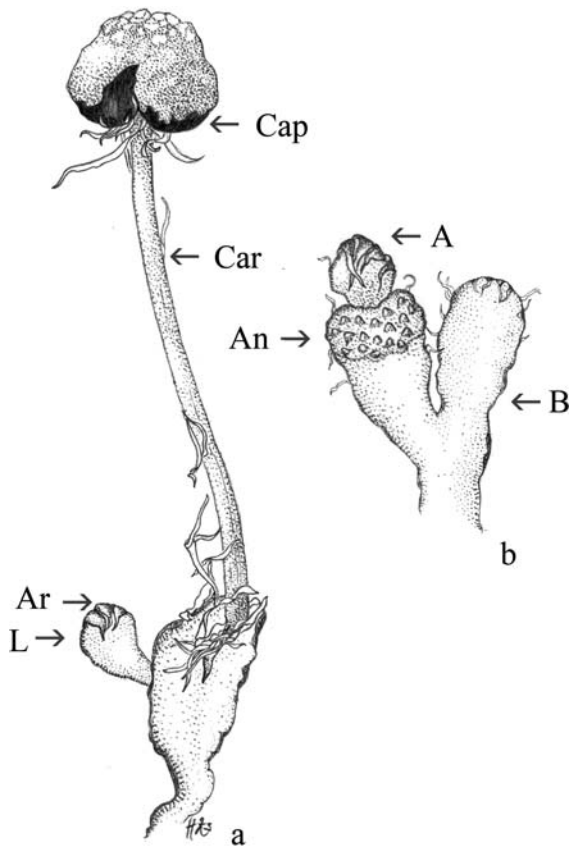

Fig. 2 Morphological traits of *Mannia fragrans*. **a**. female plant, **b**. male plant. *A* sub-apical branch, *An* antheridia, *Ar* archegonia covered by scales, *B* bifurcation, *Cap* capsule, *Car* carpophore, *L* lateral branch

decaying fragments from below this region (some ventral–lateral primordia were often observed on such parts during microscopical cleaning of plants), and 27 apical parts of female plants with opened capsules.

Data analysis

Population sex ratios were estimated for each population separately, at the following levels: pooled values for all sampling dates, sampling dates separately, and at the patch level. Since not all patches had enough samples available for statistical analysis, counts from each patch were pooled over sampling dates. Deviations from the 1:1 sex ratio were investigated with Fisher's exact test and, in case of high number of observations, the maximum likelihood chi-square test (Sokal and Rohlf 1995).

To test whether the sex state of the thalli was associated with clonal traits and patch size, a log-linear analysis was conducted (Sokal and Rohlf 1995). If two traits are associated, a significant interaction between them is expected. Significance of the interaction was tested by determining the change in the log-likelihood ratio after adding or deleting the given interaction from the model. Sex state included four categories: males, females, asexual, and bisexual plants. The number of bifurcations and number of lateral branches were divided into three classes (0, 1, $\geq$2), and the number of sub-apical branches into two classes (0, $\geq$1). For patch sizes, two categories were established: small patches containing 1–100 individuals and large patches containing more than 100 individuals. Altogether 385 plants were analyzed (93 males, 152 females, 123 asexual, and 17 bisexual plants).

To test the effect of sporophyte production on the number of bifurcations and lateral and sub-apical branches, data from 28 individuals with and 14 individuals without mature sporophytes from the second sampling date were compared using a chi-square test. We divided the different traits into the same categories as described above.

The effect of fragment type on regeneration ability was tested with a maximum likelihood chi-square test (Sokal and Rohlf 1995). All analyses described above were conducted with the SPSS software package (SPSS for Windows 11.0.1 2001).

In the following analyses each ISSR band was scored as a separate locus with two possible allelic states (band present or absent). Standard genetic indices including the number (S) and proportion of polymorphic loci (%p), occurrence of private haplotypes (i.e., restricted to one population), average gene diversity over loci (H_S, Nei 1987), and mean haplotype diversity (h_S, Nei 1987) were calculated for all populations at all sampling dates. These analyses were performed using the ARLEQUIN 3.01 software package (Excoffier et al. 2005). The number of bands and the number of private bands/population (i.e., restricted to one population) were established for each sampling date using GenAlEx 6 (Peakall and Smouse 2006).

In order to gain information about the relative importance of recombination compared to that of somatic mutation in creating genetic diversity, the incompatibility excess ratio (Wilkinson 2001) was calculated for all populations at all sampling dates using the PICA 4.0 software (Wilkinson 2001). In two binary character data, such as the presence or absence of ISSR bands at two loci, the presence of all four possible combinations of characters (0/0, 1/0, 0/1, 1/1) is more parsimoniously explained by recombination than by three mutation events assuming the infinite allele model. This is referred to as matrix incompatibility, and can be used as a measure of recombination when summed over all pairwise comparisons. In case of matrix incompatibility, the contribution of a particular genotype was calculated by jackknifing using the JACTAX option (using empirical frequencies and 1,000 randomizations) in PICA (Wilkinson 2001) to determine the proportion of unique genotypes that are likely the result of mutation, and thus are part of a clonal lineage.

To evaluate the association among loci in each population and to explore if allele distributions originate from sexual or asexual reproduction, we used an estimate of multilocus linkage disequilibrium independent of sample size (r_d), calculated by use of the Multilocus 1.2 software (Agapow and Burt 2000), and 1,000 artificially recombined data sets were used to determine the statistical significance of the test.

Results

Sex expression and population sex ratios

Sex state of thalli was significantly correlated with patch size (Table 5): large patches had more sexual

Table 4 Deviation of sex ratios from 1:1

Population/sampling	No. of ♀	No. of ♂	χ^2
Pop 1/2	25	2	13.251*
Pop 1/3	199	65	40.691*
Pop1/all	224	67	*46.578**
Pop 2/1	78	30	11.342*
Pop 2/2	129	39	26.431*
Pop 2/3	324	111	*56.304**
Pop 2/all	531	180	*92.317**
Pop 3/3	637	196	*127.757**

Results from the different populations at all sampling dates. Significance values originate from the Fisher's exact test, except for high sample numbers, where the maximum likelihood χ^2 test was performed (*italics*)

*$P < 0.001$

individuals (83%, $n = 141$) than small ones (59%, $n = 244$). Sex ratios were significantly female-biased at all sampling dates for all sites investigated (Table 4). Sex ratios of individual patches showed a similar tendency: counts of male plants were always lower than those of females in all patches in both populations. In P1, this relationship was significant in four of nine patches ($P < 0.05$) and marginally significant ($0.05 < P < 0.06$) in one patch according to the results of the Fisher's exact test. In P2 significant differences were found in 5 out of 14 patches ($P < 0.05$) and marginally significant differences in two additional patches ($0.05 < P < 0.07$). The frequency of bisexual plants was very low at all sites investigated (1–2%).

Sex-specific clonal traits

When comparing females and males alone, no difference was found in the number of bifurcations and lateral branches (Table 5). However, a significant association was found between sex state and the number of sub-apical branches, with higher numbers in males than in females (no branches: 28%, $n = 93$, and 92%, $n = 152$ for males and females, respectively). Patch size and the number of lateral branches were also associated: in smaller patches fewer such branches were formed (no lateral branches: 78%, $n = 244$, and 72%, $n = 141$ for small and large patches, respectively).

When only bisexual plants were excluded from the analysis, the same association was found between sex state and number of sub-apical branches. Additionally, significant associations were obtained between sex state and number of bifurcations and number of lateral and sub-apical branches. Asexual plants had significantly more bifurcations (plants with bifurcations: 45%, $n = 262$, and 82%, $n = 123$ for sexual and asexual plants, respectively) and significantly less sub-apical and lateral branches than males or females (plants with lateral branches: 29%, and 12%, and with sub-apical branches: 34% and 6%, for sexual and asexual plants, respectively).

Finally, analyses of all sex states, including bisexual plants, yielded an additional association between sex state and number of lateral branches. Compared to other sex states, the number of plants producing two or more lateral branches was highest in bisexual plants (47%, $n = 17$ and 10%, $n = 368$ for bisexual plants and other sex states together, respectively). Similarly, the number of bifurcations was also higher in bisexuals than in other sex states (plants with more than two bifurcations: 76%, $n = 17$ and 56%, $n = 368$ for bisexual plants and other sex states, respectively).

Significant differences between females with and without sporophytes were only found in the number of sub-apical branches, which was proportionally higher in the latter ($\chi^2 = 10.18$, df $= 2$, $P < 0.001$).

Importance of sexual versus asexual reproduction

From the cultivated fragments, green parts including the apex showed significantly higher regeneration capacity than brownish lower ones ($\chi^2 = 55.658$, df $= 1$, $P < 0.05$). Fragments of female plants with sporophytes, having shed spores immediately before sampling, showed no regeneration at all.

Of the 17 archegoniophores analyzed, 10 were genetically identical to the mother plant, and the remaining seven differed. An example of the latter is shown in Table 6. As an archegoniophore bears several sporophytes, genetic patterns differing from that of the mother plant may reflect fertilization of some archegonia by a genetically different male plant. Estimation of multilocus linkage disequilibrium (r_d) showed significant deviation from the hypothesis of free recombination (Table 7).

Table 5 Log-linear analysis of associations between the number of bifurcations, lateral and sub-apical branches, and patch size and the sex state of *Mannia fragrans*

Association tested with sex	Model	df	Pearson χ^2
No. of bifurcations (SB)	LB, AB, PB, AL, PL, PA, S	121	347.73
	LB, AB, PB, AL, PL, PA, SB	115	*273.59*
	SB	6	74*
No. of lateral branches (SL)	LB, AB, PB, AL, PL, PA, S	121	347.73
	LB, AB, PB, AL, PL, PA, LS	115	*302.39*
	LS	6	45*
No. of apical branches (SA)	LB, AB, PB, AL, PL, PA, S	121	347.73
	LB, AB, PB, AL, PL, PA, AS	118	*171.84*
	AS	3	176*
Patch size (SP)	LB, AB, PB, AL, PL, PA, S	121	347.73
	LB, AB, PB, AL, PL, PA, SP	118	*333.83*
	SP	3	14*
No. of bifurcations with sex (SB)	LB, AB, PB, AL, PL, PA, SL, SA, SP	109	138.36
	LB, AB, PB, AL, PL, PA, SL, SA, SP, SB	103	*86.563*
	SB	6	52*
No. of lateral branches with sex (SL)	LB, AB, PB, AL, PL, PA, SB, SA, SP	109	106.7
	LB, AB, PB, AL, PL, PA, SA, SP, SB, SL	103	*86.563*
	SL	6	20*
No. of apical branches with sex (SA)	LB, AB, PB, AL, PL, PA, SB, SL, SP	106	210.58
	LB, AB, PB, AL, PL, PA, SP, SB, SL, SA	103	*86.563*
	SA	3	124*
Patch size with sex (SP)	LB, AB, PB, AL, PL, PA, SB, SL, SA	106	99.3*
	LB, AB, PB, AL, PL, PA, SB, SL, SA, SP	103	86.563
	SP	3	

Statistical significance of each interaction was tested by determining the change in the log-likelihood ratio after adding (italicized models) or deleting (non-italicized models) that specific interaction from the model. Associations tested are given in brackets. *S* sex state (1, 2, 3, 4), *B* no. of bifurcations, *L* no. of lateral branches, *A* no. of sub-apical branches, *P* patch size. $*P < 0.05$. None of the three or higher order interactions were significant. When excluding bisexual or bisexual and sterile plants from the analysis, the following interactions were found to be significant ($P < 0.05$): SB, SA, SP, AL and B, PL, SA, respectively

Table 6 Results of the simultaneous genetic analysis of sporophytes and mother plants

♂	0	0	***1***	1	***1***	0	1	0	1	0	1	0
♀	1	0	0	1	0	1	1	1	1	0	1	0
Sp	1	**1**	***1***	1	***1***	1	1	1	1	**1**	1	**1**

Example of plants originating from patch 3. *Sp* sporophyte, ***1*** = fragment found in male plants from the same patch, **1** = fragment found in male plants from other patches

Significant matrix incompatibility was found only in two cases (Table 7), and according to the JACTAX algorithm, the number of genotypes accounting for this incompatibility and hence very likely derived from recombination was very low.

Genetic diversity within populations

The genetic characteristics of the studied populations are summarized in Table 7. On average approximately half of the loci were polymorphic; Nei's gene diversity was 0.059 and haplotype diversity values were 0.150. The number of genets compared to the number of ramets sampled was relatively low, mean haplotype numbers being 12.7, 12.0, and 5 for P1, P2, and P3, respectively. In addition, about half of these haplotypes were rare and many of the observed haplotypes differed in only one mutation. Almost all populations had private bands and the proportion of private haplotypes was high attaining 22.43 on average.

Table 7 Genetic variability in different populations of *Mannia fragrans*

Population/sampling	No. of s/no. of h	S	%p	No. of bands/no. of private bands	No. of bands under 5% freq.	No. of private haplotypes	No. of haplotypes under 5% freq.	HS ± SD	h_S ± SD	IER	r_d
Pop1/1	43/12	50	79.4	55/19	31	6	6	0.075 ± 0.042	0.147 ± 0.097	0.251*	0.554*
Pop1/2	34/13	35	66.0	43/15	0	6	9	0.099 ± 0.055	0.139 ± 0.092	0.663*	0.692*
Pop1/3	44/13	35	76.1	44/16	18	7	9	0.074 ± 0.043	0.136 ± 0.089	0.081	0.454*
Pop2/1	45/13	37	58.7	45/8	20	7	7	0.055 ± 0.032	0.126 ± 0.084	0.005	0.450*
Pop2/2	44/13	24	45.3	38/10	14	9	9	0.049 ± 0.030	0.126 ± 0.084	0.603*	0.119*
Pop2/3	59/10	9	19.6	28/2	3	6	6	0.040 ± 0.026	0.157 ± 0.105	0.639*	0.105*
Pop3/3	41/5	7	15.2	27/0	3	2	2	0.021 ± 0.016	0.218 ± 0.162	1.000*	0.178*

No. of s/No. of h = number of samples/number of haplotypes; S = number of polymorphic loci; %p = percentage of polymorphic loci; H_S = average gene diversity over loci; h_S = average haplotype diversity; IER = incompatibility excess ratio; r_d = multilocus linkage disequilibrium; $^*P < 0.005$. Number of private bands/haplotypes: bands/haplotypes restricted to one population

Discussion

Sex expression

The overall rate of sex expression observed in this study (0.84, based on counts from 1,350 plants) is high, but falls within the range of values obtained for other species (Bisang and Hedenäs 2005).

Gametangial induction, often resulting from the complex interaction of numerous factors, is poorly studied in bryophytes. The positive correlation observed between patch size and sex expression observed is not unique; a similar relationship was found in *Marchantia inflexa* (McLetchie and Puterbaugh 2000). However, the underlying causes are probably not the same. Whereas in *Marchantia*, increased sex expression in larger patches was associated with the higher diversity of microhabitats, no such differences exist in *Mannia* due to the substantially smaller size of patches. Enhanced sex expression in larger patches is probably the result of the interaction of several factors. First, large patches represent older colonies based on the several layers of dead plants found below them, which is lacking in small patches. Hence, higher rates of sex expression could simply be related to the longer time period available for reaching maturity. In small patches, representing an early colonization stage, space limitation is lacking; thus germinating plants first invest into growth. Additionally, as long as enough space is available, plants continue to grow and to branch (Damsholt 2002 and high number of bifurcations found in asexual plants from smaller patches). Second, sex expression may also be stimulated by the contact with other plants (Kimmerer 1994). In *Mannia*, crowding is suggested to put an end to dichotomous branching and to induce production of intercalary branches (Damsholt 2002), which usually then develop sex organs. Whether this is induced by some chemicals as in ferns and some bryophytes (Fernandez et al. 1997; Chopra and Sood 1973) needs further investigation. Finally, a positive influence of hydration on sex expression is also plausible (Kumra and Chopra 1983) through the better retention of water in large patches due to the densely packed, concave thalli and the vaste rhizoid net below them.

Sex ratio and sex-specific clonal traits

As in the majority of dioicous bryophytes (Bisang and Hedenäs 2005), sex ratios were strongly female-biased

in *Mannia fragrans*. The observed F:M = 3:1 ratio is within the range reported for other dioicous species with female predominance (Bisang and Hedenäs 2005). Male plants invested more in clonal growth: according to former observations (Hock 2007), in contrast to females, where sex organs were produced on lateral branches, male organs usually terminated leading branches and lateral branches were generally sterile. Moreover, males produced considerably more sub-apical branches, often developing new antheridia in the following season. While production of antheridia in *Mannia* does not involve the apical cell, allowing continuous growth (Haupt 1921), archegoniophores arise from the apical cell, stopping apical growth (Leitgeb 1881). Sub-apical growth following sporophyte production is rare (low percentages of sub-apical branches and lack of regeneration from apical fragments in females with mature gametophores); it mainly occurs in cases of sporophyte abortion and involves the formation of a new growing point (Haupt 1929 and pers. obs.). A differential cost of producing organs of the two sexes may explain the differences in subsequent clonal growth between them. Experimental approaches of differences in the cost of realized sex expression between sexes are sparse and do not allow for generalizations (Bisang and Hedenäs 2005). However, it can be postulated that in fertilized individuals of *Mannia*, the production of stalked female gametophores and the large spores may need more energy than that of sessile antheridia. Yet, if it is so, the differential cost of producing gametangia of the two sexes does not explain the observed female-biased sex ratios. Sex-specific survival or tolerance (McLetchie and Puterbaugh 2000; Stark et al. 2001) can also be excluded, since the two sexes grow intermingled. Regulation by chemical factors may be plausible (Bhatla and Chopra 1981); however, based on field observations, it seems more likely that sex expression is labile and dependent on seasons, age of plants, or other environmental factors (Wyatt and Anderson 1984; Korpelainen1998). It is also likely that the production of male and female organs is sequential, as suggested in the Polytrichaceae (Glime 2007). If this is the case, frequency-dependent selection is responsible for eliminating the surplus of males (Fisher 1930).

If resources are limited, a fertilized female is expected to have fewer resources for clonal growth than an unfertilized one (Stark et al. 2001). Higher numbers of sub-apical branches in females with no or aborted sporophytes found in this study are consistent with this hypothesis. However, it may be hard to differentiate between the effect of apical dominance on the production of sub-apical branches and a potential cost of reproduction (Stark 2002b).

Low frequency of bisexual thalli in the populations investigated is comparable to patterns observed in *Preissia quadrata*, a liverwort with similar life-history traits (Haupt 1926). This rarity raises several intriguing questions needing further investigation. Why are such individuals so rare? What is the advantage of maintaining them in the populations and how are they maintained? To date, little is known about sex determination in bryophytes (Ramsay and Berrie 1982). Monoecy is supposed to be associated with diploidy or polyploidy of gametophytes, whereas dioecy with a haploid chromosomal set (Wyatt 1994), provided that sex determination is under genetic control. However, this is not necessarily so, as environmental factors or plant condition may also influence it (Korpelainen 1998), which is probably the case in *Mannia* as well. The fact that bisexual and asexual plants mostly occurred in small, newly colonized patches (pers. obs.) and that their clonal traits and size were also very similar to each other suggests that selection may prefer colonizers that develop sexual organs of both sexes, hence ensuring sexual reproduction when potential partners are not present. Since to date plants failed to produce gametangia in culture, long-term in situ monitoring of the sexual condition and dynamics of individual plants may provide a tool for revealing exact details about this process. As an alternative, bisexual plants may also represent rare diploids in populations mainly consisting of haploid unisexuals, though chromosome counts of the species are uniformly 9 (Fritsch 1991). For this reason, it is more probable that the species is genetically bisexual as proposed by Schuster (1992) and that the expression of both sexes or only one sex is determined by some environmental factor. Since the species is reported to be polyoicous or unisexual (Damsholt 2002), it would be worth investigating whether the ratio of bisexual plants varies with age of populations or geography, to elucidate the role of these individuals in the evolution of populations.

Asexual propagation by fragmentation is very effective in *Mannia fragrans*. Cultivation experiments show that apical fractions of thalli containing

meristematic tips are most likely to survive periods of unfavorable conditions. New branches mainly arose from the lateral ventral region of the thalli. Similar patterns were found in a closely related species, *Asterella californica*, living in areas with comparably arid summers (Haupt 1929).

Reproductive characteristics and their footprints in population genetics

General trends in the partitioning of genetic diversity differ between the two major groups of bryophytes. Compared to mosses, overall level of intrapopulation genetic diversity is assumed to be relatively low in liverworts especially in simple and complex thalloids (Wyatt 1994). Underlying factors, including wider ecological amplitude of mosses and reduced capacity for sexual reproduction of liverworts (Khanna 1964), have been debated but it is still not clear why liverworts should behave differently. Average gene diversity in *Mannia* was also low compared to the majority of mosses but falls into the range described for liverworts and very close to that of species with similar reproductive characteristics (Szweykowski and Zielinski 1983; Boisselier-Dubayle and Bischler 1997).

A unisexual, outbreeding species is expected to show greater levels of genetic diversity than a bisexual, inbreeding one (Loveless and Hamrick 1984). Similar life-history characteristics and habitat preferences may predict similar genetic patterns, but this is not always necessarily so (Wyatt et al. 1989; Dewey 1989; Stenøien and Såstad 2001). Although most liverworts are unisexual (Wyatt and Anderson 1984), they usually show little or no genetic variation (e.g., Dewey 1989; Bischler and Boisselier-Dubayle 1993). Low levels of genetic variation were observed in the predominantly unisexual *Mannia fragrans* as well. In *Preissia quadrata*, a species with similar spore size and reproductive characteristics (Boisselier-Dubayle and Bischler 1997), low genetic variation was attributed to predominant asexual reproduction. Fertilization and subsequent recombination provide a possibility for creating new genetic combinations. Yet, intra-gametophytic selfing and crossing between genetically identical clones result in spores genetically equivalent to asexual propagules (Wyatt et al. 1989). Given the rarity of bisexual *Mannia* thalli, the role of intra-gametophytic selfing is probably negligible. If individual patches are not genetically uniform, outcrossing between different genotypes may occur, as indicated by the results of this study. Though given that only a few haplotypes, very often differing in one mutation only, dominate the populations, the number of possible combinations among them is restricted; hence recombination does not necessarily lead to an increase in new haplotypes. For this reason, populations are composed of only a few clones. The presence of rare alleles and numerous rare recombinant haplotypes shows that in some cases recombination and mutation do give rise to new haplotypes, increasing within-population diversity. The probability that these haplotypes represent spores coming from remote localities is low due to the large spore size of the species and the great distance between the isolated populations (Hock et al. 2008). New haplotypes have, however, little chance to spread effectively, given that most spores fall into their patch of origin where there is little possibility for germination due to the densely packed thalli. As populations are rather small and isolated, the effect of genetic drift is enhanced, which probably leads to continuous elimination of the rare new haplotypes, though some of them may be conserved in the diaspore bank (Hock et al. in prep.). The above-mentioned hypotheses are consistent with the obtained r_d values showing very high linkage among loci compared to other species (Hassel et al. 2005; Gunnarsson et al. 2005), which suggests rare recombination events and dominance of asexual elements of the reproduction in spite of regular sporophyte formation (83% of sexual females built sporophytes).

The comparison of the role of sexual and asexual elements of the reproductive system in the functioning of the populations showed that although both reproductive modes are frequent, the asexual elements play a greater role in shaping the genetic structure of the populations. Furthermore, new information has been obtained on the functioning of the poorly investigated polyoicous reproductive system in bryophytes. The present results on the reproductive biology of *Mannia fragrans* show that investigation of the dynamics and functioning of the reproductive system in polyoicous bryophytes is of particular interest. It would be especially intriguing to determine the importance of the coexistence and the relative role of the different sexual states in other polyoicous species to be able to draw general trends.

Investigating the variation in sexual expression (male, female, bisexual) with time would be of great interest to detect whether patterns similar to sequential hermaphroditism in animals may exist in bryophytes.

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